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# Prepared and screened a modified TNF-α molecule as TNF-α autovaccine to treat LPS induced endotoxic shock and TNF-α induced cachexia in mouse

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**Abstract**

Overexpression of TNF-α in the body is critically involved in many diseases. A strategy to construct TNF-α autovaccine by introducing a T cell helper epitope to the protein has been developed and may be an alternative because it is cheaper and highly efficient. However, the induction of high level anti-TNF-α neutralizing autoantibodies by TNF-α autovaccine is depend on a proper T cell help epitope. In order to evaluate the effect of different T helper cell epitopes on the immunogenicity of mouse TNF-α (mTNF-α), three T helper cell epitopes, TT (QYIKANSKFIGITEL), HEL (NTDGSTDYGILQINSR), and PADRE (AKFVAAWTLKA), were chosen for this study. The sequence (amino acids 126–140) of mTNF-α was replaced with those of the T cell help epitopes, respectively. The three fusion proteins (mTNF-TT, mTNF-HEL, mTNF-PADRE) were expressed in *Escherichia coli* and purified with a simple strategy. The abilities of the proteins elicited TNF-α autoantibodies in BALB/c mice were investigated. The results showed that mTNF-PADRE is the most effective among the three modified TNF-α molecules. In the absence of adjuvant, the therapeutic effect of TNF-PADRE on LPS induced endotoxic shock mice and mTNF-α induced cachexia mice was observed. This study suggests that mTNF-PADRE may be a better candidate of mTNF-α autovaccine.
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*Keywords:* TNF-α; Autovaccine; T cell helper epitope; Cachexia; Endotoxic shock; Adjuvant

## 1. Introduction

TNF-α is an important proinflammatory cytokine involved in normal physiological immune and inflammatory processes. However, TNF-α also induces chronic inflammation and subsequent tissue destruction, such as cachexia [1,2], Crohn's disease [3,4], and rheumatoid arthritis [5,6] when it is overexpressed. More recently, inappropriately expressed TNF-α was also shown to play a role in the development of cancer [7,8]. It was demonstrated that blocking of TNF-α could reduce the symptoms of inflammatory disease [9,10]. Some anti-TNF-α monoclonal antibodies [11,12] and the soluble TNF-α receptor [13] have been approved for clinical use as TNF-α antagonists and successfully relieved pain and symptoms in patients suffering from rheumatoid arthritis and Crohn's disease. However, the long-term use of monoclonal antibodies and engineered receptors could cause some problems, such as a variable degree of immunogenicity and major costs [14–16].

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A strategy to construct protein autovaccine by introducing a T helper cell epitope to the protein has been developed [17]. A mouse TNF-α (mTNF-α) autovaccine was constructed by the strategy and investigated. The result demonstrated that the antibodies against mTNF-α were elicited after mice were injected with modified mTNF-α. The symptoms of experimental cachexia and type II collagen-induced arthritis in mice were markedly ameliorated [17]. The efficacy between the anti-TNF monoclonal antibodies and the mTNF autovaccine on reducing metastasis in mice was also compared [18]. The result showed that the both were successful in reducing the number and size of lung metastases without difference.

The evidence has showed that immunogenicity of TNF-α protein autovaccine was related with the native structure of the protein after it is purified from *Escherichia coli* [20]. In addition, type of T helper cell epitope also affects the titer of the protein autovaccine [19]. Consequently, it was important to choose a proper T helper cell epitope and develop effective purification strategy for obtaining a proper TNF-α protein autovaccine.

In this study, we constructed three recombinant mTNF-α mutants by introducing three T cell helper epitopes (TT, HEL, and PADRE) to mTNF-α, respectively. A simple purification process was developed. BALB/c mice were immunized with the three recombinant vaccines. Our results indicated that mTNF-PADRE is most effective to elicit anti-mTNF-α neutralizing autoantibodies among the three proteins in vivo. In the absence of adjuvant, the therapeutic effect of TNF-PADRE was observed on the cachexia mice or endotoxic shock mice.

## 2. Materials and methods

### 2.1. Materials and reagents

Restriction endonucleases, *Taq* polymerase and T4 DNA ligase were purchased from Takara (Dalian, China). Urea, guanidine hydrochloride, β-mercaptoethanol, Triton X-100, and Tris were purchased from Serva (Germany). Sephacryl-100 and Sephacryl-300 resins were purchased from Amersham Phamacia Biotech (Wikströms, Sweden). LPS, complete Freund's adjuvant and incomplete Freund's adjuvant were purchased from Sigma (Saint Louis, Missouri, USA). mTNF-α for ELISA was purchased from Pepro Tech, Inc. (Rocky Hill, NJ, USA) and mTNF-α for immunization and cachexia mice induction was purified by our laboratory. Purity is above 95% and bioactivity is $4 \times 10^6$ IU/mg. Goat anti-mouse TNF-α polyclonal antibody was purchased from Santa Cruz Biotechnology (California, USA) and raised against a peptide mapping at the N terminus of TNF-α of mouse origin. Rabbit anti-goat IgG alkaline phosphatase conjugate was purchased from Zhongshan Golden Bridge Biotechnology Co. Ltd. (Peking, China). Horseradish peroxidase-labeled goat anti-mouse IgG (Zhongshan Golden Bridge Biotechnology Co. Ltd. (Peking, China). The plasmid pBV220 containing the PR and PL promoters, the clts857 gene, and two strong transcription terminators was made in home [21]. Native mTNF-α gene was synthesized by Shenggong (Shanhai, China). All other chemicals were of analytical grade.

### 2.2. Construction, expression, and purification of recombinant mutant mTNF-α molecules

The genes of all three recombinant mutant mTNF-α molecules were constructed by PCR and gene synthesis. The sequence of three recombinant genes was designed to include EcoRI, BglII, NdeI, and SalI restriction sites. The sequence from EcoRI site to BglII site is mTNF (amino acid residues: 1–125). The sequence from BglII site to NdeI site is TT, HEL, or PADRE sequence. The sequence from site NdeI to SalI site is mTNF-α (amino acid residues: 141–156). The three genes were all cloned into pBV220 vector from EcoRI and SalI sites, identified using DNA sequencing and the final plasmids were named as pBV220-mTNF-TT, pBV220-mTNF-HEL, and pBV220-mTNF-PADRE, respectively, (Fig. 1). *Escherichia coli* DH5α cells were transformed with the three recombinant plasmids.

A DH5α clone transformed with pBV220-mTNF-TT, pBV220-mTNF-HEL, or pBV220-mTNF-PADRE was used to inoculate 10 ml Luria-Bertani (LB) medium supplemented with ampicillin (100 μg/ml) grown with 200 rpm shaking overnight at 30 °C, respectively. Three milliliters of culture were transferred to 300 ml fresh LB medium in a 500 ml shake flask. The culture was grown with 200 rpm shaking at 30 °C until the $OD_{600}$ reached 0.5 and induced by changing temperature from 30 to 42 °C. After incubation at 42 °C for 4 h, 1 ml of culture was collected and analyzed by electrophoresis on a 15% polyacrylamide–sodium dodecyl sulfate gel (SDS–PAGE), and stained by Coomassie blue R-250.

Fermentation was performed using the fermentor (working volume of 5 L) as described [22]. The induced bacteria were harvested by centrifugation. Ten grams (wet weight) of the harvested cell paste were sonicated in ice. The pellet was collected by centrifugation. Inclusion body of mTNF-HEL washed with 100 ml of 4 M urea, 1% Triton X-100, 5 mM EDTA, 5 mM β-mercaptoethanol, 50 mM Tris–HCl pH 8.5 at room temperature for 30 min twice, followed with 100 ml of 5 mM EDTA, 50 mM Tris pH 8.5 at room temperature for 30 min. Inclusion bodies of TNF-TT and TNF-PADRE washed with 100 ml of 4 M urea, 1% Triton X-100, 5 mM EDTA, 5 mM β-mercaptoethanol, 50 mM Tris–HCl pH 8.5 at room temperature for 30 min twice, followed with 100 ml of 6 M urea, 5 M EDTA, 50 mM Tris pH 8.5 at room temperature for 30 min. The final pellets of inclusion bodies were solubilized by homogenizing in 20 mM β-mercaptoethanol, 7 M guanidine hydrochloride. The supernatant was collected by centrifugation. A chromatography column ($2.4 \times 70$ cm) was packed with 350 ml of depyrogenated Sephacryl-100 resin for the purification of TNF-TT. The

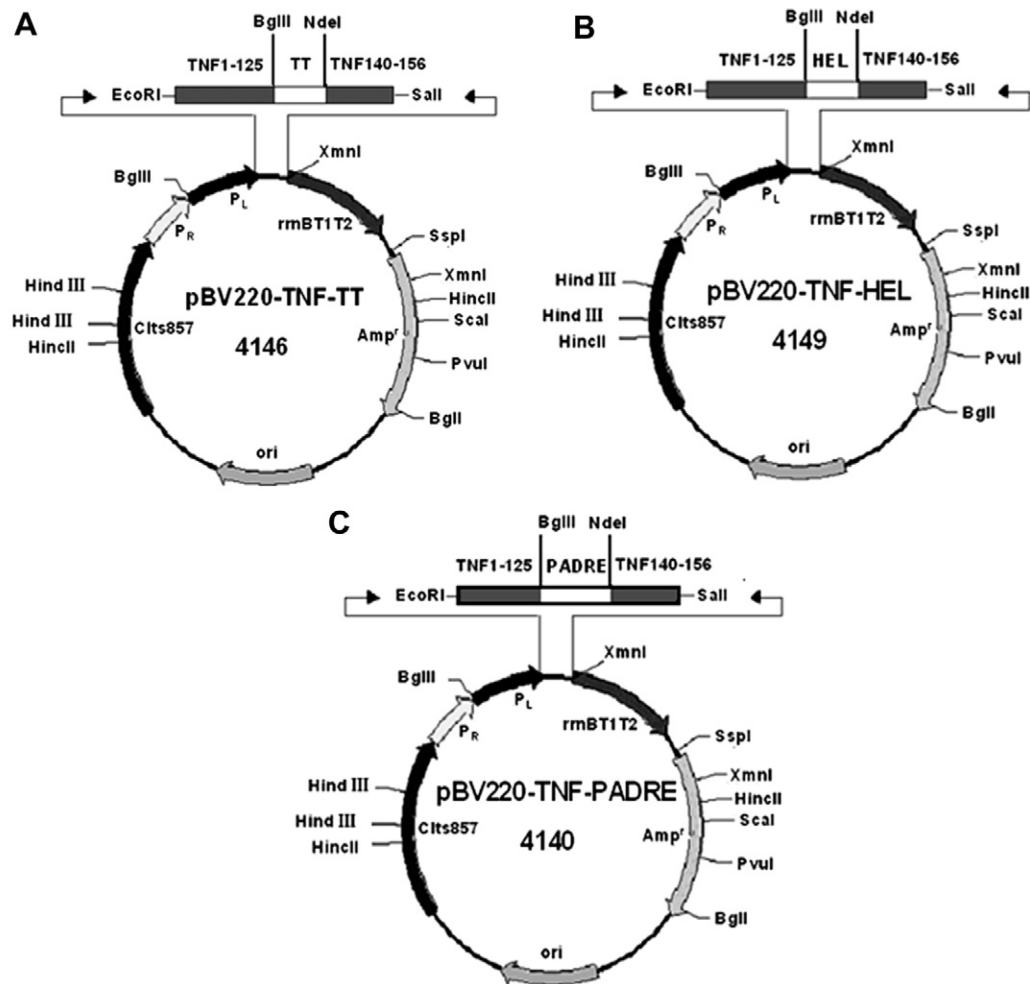


Fig. 1. Maps of mTNF mutants. (A) Map of pBV220-TNF-TT expression vector. (B) Map of pBV220-TNF-HEL expression vector. (C) Map of pBV220-TNF-PADRE expression vector.

column was equilibrated with 50 mM Tris–Cl, pH 8.5, 8 M urea, and 150 mM NaCl. The supernatant of solubilized mTNF-TT was applied to the column. The chromatography was performed at a flow rate of 0.3 ml/min. mTNF-TT fraction was eluted with equilibration buffer. Another chromatography column ($2.4 \times 100$ cm) was packed with 500 ml of depyrogenated Sephacryl-300 resin for the purification of mTNF-HEL and mTNF-PADRE at a flow rate of 0.5 ml/min using above equilibration buffer. The purified proteins (0.2 mg/ml) were dialyzed against refolding buffers composed of 10 mM Tris–HCl, pH 8.5 supplement with 1/1000 (v/v) β-mercaptoethanol, $1/10^5$ (v/v) 0.5M CuSO4 at 20 °C for 8 h. Then purified proteins were dialysised against $H_2O$ at 20 °C for 8 h twice. The refolded proteins were collected by centrifugation to remove the pellet. The final renatured proteins were stored at −70 °C.

### 2.3. Characterization of recombinant mutant mTNF-α molecules

SEC-HPLC analyses were performed on a Beckman's HPLC system. The samples in PBS were injected onto a $7.5 \times 300$ mm G2000SW column (TOSOH) at a flow rate of 0.5 ml/min. Peaks were detected by monitoring at a wavelength of 280 nm. The purity of proteins was calculated as a percentage of the total peak area detected.

Proteins were transferred to nitrocellulose membranes (0.22 μm; Invitrogen, USA) after SDS–PAGE using a Bio-Rad Trans-Blot Semi-Dry electrophoretic cell. Western blot analyses were carried out using an mTNF-α polyclonal antibody, followed by an alkaline phosphatase-labeled IgG. Western Blue Stabilized Substrate (Promega) for alkaline phosphatase was used for detection.

The cytolytic activity of purified mTNF-TT, mTNF-HEL, and mTNF-PADRE proteins was determined on L929 cell line [23]. Murine fibroblast L929 cells in DMEM supplemented with 10% fetal calf serum (FCS) were seeded in 96-well plates at $1 \times 10^5$ cells/ml and incubated for 16 h. The mTNF-TT, mTNF-HEL, or mTNF-PADRE protein (100 μg/ml) was serially diluted with DMEM supplemented with 3% FBS and 0.35 μg/ml actinomycin D (Sigma). The diluted proteins were added to the cells (100 μl/per well) and incubated for 16 h at 37 °C and 5% $CO_2$. The cell supernatant was removed thereaf-

ter and the monolayer stained with 0.05% crystal violet in 20% ethanol in room temperature for 5 min. Cells were rinsed gently with distilled water, and the bound dye was eluted from survival cells with 100 μl/well of 0.1% acetic acid in 50% ethanol. The absorbance of washed stained cell monolayer was measured at a wavelength of 570 nm using an automatic plate reader (Bio-Rad, Hercules, CA). Saline was negative control and recombinant mTNF-α was positive control.

### 2.4. Immunization of animals and detection of anti-mTNF-α serum antibodies

Healthy male BALB/c mice (5-weeks-old, purchased from the National Rodent Laboratory Animal Resource, Shanghai, China) were cared for under institutional animal care protocols. Antisera were prepared by inoculating BALB/c mice with 30 μg of mTNF-TT, mTNF-HEL, or mTNF-TT in Freund's adjuvant. Every group mice ($n = 5$) were inoculated subcutaneously four times at 2-week intervals. MTNF-TT, mTNF-HEL, or mTNF-TT was dissolved in sterile saline and diluted with complete Freund's adjuvant at 1:1 for the initial injection, and diluted with incomplete Freund's adjuvant at 1:1 for subsequent inoculations. Mice ($n = 5$) immunized with PBS or mTNF-α diluted with complete Freund's adjuvant at 1:1 or incomplete Freund's adjuvant were established as control. Serum samples were collected 2 weeks after the final boost.

Specific serum binding antibodies against mTNF-α were detected by ELISA. Ninety-six-well ELISA plates were coated with 50 ng/well of the recombinant mTNF-α protein. Obtained serum was diluted $1 \times 10^3$ and $1 \times 10^4$-fold in phosphate-buffered saline (PBS)–0.5% BSA and applied to wells at room temperature for 2.5 h. The association of the serum with mTNF-α was determined by using horseradish peroxidase-labeled goat anti-mouse IgG at 1:1000 as a secondary antibody (incubated at room temperature for 1 h). Upon development, the optical densities at 490 nm were read by a Thermo Max microplate reader (Molecular Devices Corp., Sunnyvale, CA). $OD_{490}$ values that were greater than twice that of negative serum were considered positive.

Specific serum neutralizing antibodies against mTNF-α were detected by assay for neutralization of TNF-α-mediated cytotoxicity using serum antibodies. As described [23], before beginning the assay, 100 μl/well of murine fibroblast L929 cells in DMEM supplemented with 10% fetal calf serum (FCS) were seeded in 96-well plates at $1 \times 10^5$ cells/ml and incubated for 16 h. The obtained serum were diluted with medium containing actinomycin D (0.35 μg/ml) and incubated with mTNF-α (20 U/ml) at 37 °C for 2 h. After removing of the supernatants of the cultured L929 cells, the mixture of serum and mTNF were separately added to the plates of the cells. Then the plates were incubated at 37 °C for 16 h, and the supernatants were removed again. Crystal violet (0.05%) in 20% ethanol was added and incubated in room temperature for 5 min. Cells were rinsed gently with distilled water, and the bound dye was eluted from survival cells with 100 μl/well of 0.1% acetic acid in 50% ethanol. The absorbance of washed stained cell monolayer was measured at a wavelength of 570 nm using an automatic plate reader. Blank control (culture alone), positive control (mixture of goat anti-mouse TNF-α polyclonal antibody and mTNF-α), mTNF-α control (mTNF-α alone), and serum control (serum alone) were also designed in the experiment.

### 2.5. Treatment with TNF-PADRE

In order to investigate whether TNF-PADRE elicited TNF-α antibody was able to neutralize mTNF-α in vivo, experimental cachexia model [17] and endotoxic shock model [24] were established as follows. A group BALB/c mice ($n = 10$) were inoculated with mTNF-PADRE (30 μg) without adjuvant subcutaneously four times at 2-week intervals. In the process, another group BALB/c mice ($n = 10$) were immunized with saline as a control. Two weeks after the last immunization, specific serum antibodies against mTNF-α were detected by ELISA. Appropriate dose ($2 \times 10^5$ IU) of mTNF-α was injected intraperitoneally daily in all mice for inducing cachexia. The survival rates of surviving mice were determined. Immunization process was as above described. After detection of specific serum antibodies against mTNF-α, appropriate dose ($1 \times 10^5$ IU) of mTNF-α was injected intraperitoneally daily in all mice. The body weights of mice were monitored.

After immunization, 600 μg of LPS was injected intraperitoneally in every BALB/c mice immunized by mTNF-PADRE or saline. In this experiment, antiserum to mTNF-α was designed as positive control and obtained as Beutler described [25]. A New Zealand White male rabbit was injected at multiple subcutaneous sites with 40 μg of mTNF-α with Freund's complete adjuvant. Three additional injections with 40 μg mTNF-α with Freund's incomplete adjuvant were given at seven days intervals. Blood was withdrawn from the rabbit seven days after the final injection. Anti-mTNF-α antibodies were detected by ELISA and 200 μl immune serum diluted 1:4 was injected intraperitoneally in BALB/c mice ($n = 10$). After 3 h, 600 μg of LPS was injected intraperitoneally in every BALB/c mouse. The survival rates of the three group mice were determined.

### 2.6. Statistical analysis

Statistical significance for differences in body weights and anti-mTNF-α serum antibodies were based on Student's two-tailed test. Significance differences in overall survival were evaluated using the log-rank test. Differences were considered significant when the $P$-value was less than 0.05.

## 3. Results

### 3.1. Preparation of antigens

The recombinant mTNF-TT, mTNF-HEL, and mTNF-PADRE were cloned into pBV220 plasmid. DNA sequence analysis conformed that the sequences of mTNF-TT, mTNF-HEL, and mTNF-PADRE genes were in agreement with the sequences that we designed.

The expression of mTNF-TT (17.8 kDa), mTNF-HEL (17.8 kDa), and mTNF-PADRE (17.4 kDa) were identified by SDS–PAGE. (Fig. 2). After inclusion bodies were washed three times, the inclusion bodies were solubilized with 7 M guanidine hydrochloride, 20 mM β-mercaptoethanol at 4 °C overnight. Approximately 80% inclusion bodies were dissolved. The soluble mTNF-TT (Fig. 3B1, lane 2) mTNF-HEL (Fig. 3B2, lane 2) and mTNF-PADRE (Fig. 3B3, lane 2) were purified by one-step Sephacryl S-100 column or Sephacryl S-300 column (Fig. 3A1, A2, and A3) and analyzed by SDS–PAGE(Fig. 3B1, B2, and B3). The purified protein was refolded by dialysis against with pH 8.5 10 mM Tris–HCl supplement with 1/1000 (v/v) β-mercaptoethanol, $1/10^5$ (v/v) CuSO4 and analyzed by SDS–PAGE (Fig. 3B1, lane 5; B2, lane 6; B3, lane 5). The final yields were 4 mg purified antigen proteins per gram of cell paste.

### 3.2. Characterization of recombinant mutant mTNF-α molecules

The purity of final purified mTNF-TT, mTNF-HEL, and mTNF-PADRE was analyzed by HPLC. All of them were >95% (Fig. 4A1, A2, and A3). Western blot proved that mTNF-TT, mTNF-HEL, and mTNF-PADRE could be recognized by goat anti-mouse TNF-α antibody (Fig. 4B).

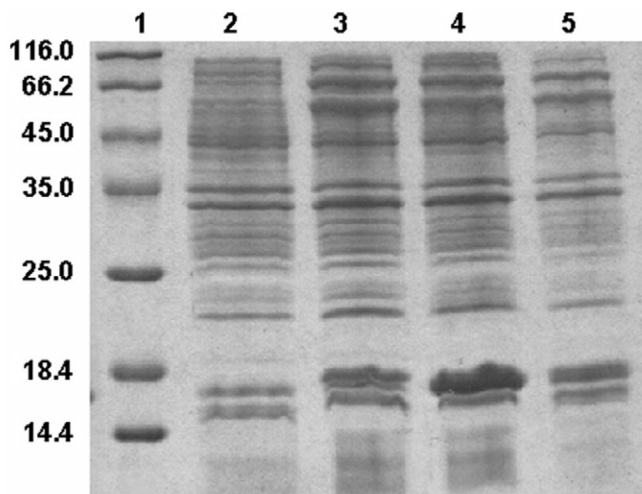


Fig. 2. Expression of mTNF-TT, mTNF-HEL, and mTNF-PADRE in *E. coli*. Lane 1, molecular weight standards (kDa); lane 2, whole cell lysate of pBV220-mTNF-TT/DH5α before induction; lane 3, whole cell lysate of pBV220-mTNF-TT/DH5α after induction; lane 4, whole cell lysate of pBV220-mTNF-HEL/DH5α after induction; lane 5, whole cell lysate of pBV220-mTNF-PADRE/DH5α after induction.

The cytotoxicity of mTNF-TT, mTNF-HEL, and mTNF-PADRE was evaluated by a standard cytolytic assay with L929 cell line. L929 cells were susceptible to the toxic effects at 1 pg/ml of native mTNF (positive control), whereas not at 100 μg/ml of the three proteins or saline (negative control) (Fig. 4C). The results showed that the three proteins lost the cytotoxicity activity of native mTNF-α.

### 3.3. Comparison of the antibody responses induced by mTNF-TT, mTNF-HEL, and mTNF-PADRE

The immunogenicity of TNF-TT, mTNF-HEL, and mTNF-PADRE were evaluated in BALB/c mice. Antibody responses against mTNF-α were evaluated by ELISA and neutralization assay of TNF-α induced cytotoxicity.

Specific serum binding antibodies against mTNF-α were detected by ELISA. As can be seen in Fig. 5A, Sera from the vaccinated mice demonstrated high-titer (more than 1:10,000) specific antibodies against mTNF-α. The mTNF-PADRE induced the strongest anti-mTNF-α antibody response among the three proteins (from 1000- to 10,000-fold dilution, TNF-PADRE group $P < 0.05$ vs. TNF-TT group and $P < 0.01$ vs. TNF-HEL group).

Next, we compared the protect abilities of L929 cells against mTNF-α induced cytotoxicity among three antiserum from the mice immunized with TNF-TT, mTNF-HEL, and mTNF-PADRE (Fig. 5B). The results showed that L929 cells were sensitive to the cytotoxic effects of mTNF-α. The mTNF-α (20 U/ml) almost induced L929 cells death completely (data not shown). Fifty micrograms per milliliter of goat anti-mouse TNF-α polyclonal antibody (positive control) can protect 64% L929 cells against 20 U/ml of mTNF-α induced cytotoxicity (data not shown). After 2-fold dilution, the antiserum induced by mTNF-TT, mTNF-HEL, and mTNF-PADRE could protect 66%, 59%, and 82% L929 cells from cytotoxicity induced by mTNF-α (20 U/ml), respectively. The antiserum induced by three proteins could neutralize mTNF-α (20 U/ml) in dose-dependent manner. The antiserum induced by mTNF-PADRE showed the highest neutralizing ability (at 2- and 4-fold dilution, mTNF-PADRE group $P < 0.05$ vs. mTNF-TT and $P < 0.01$ vs. mTNF-HEL group.).

### 3.4. Therapeutic effect of TNF-PADRE in mTNF induced cachexia mice and LPS induced endotoxic shock mice

Mice immunized with TNF-PADRE without adjuvant still rapidly developed high-titer anti-TNF-α antibody responses (more than 1:10,000) (Fig. 6A). Rabbit immunized with mTNF-α also developed high-titer anti-TNF-α antibody responses.

Protective effect of TNF-PADRE on the endotoxic shocked mice induced with LPS was evaluated in BALB/c

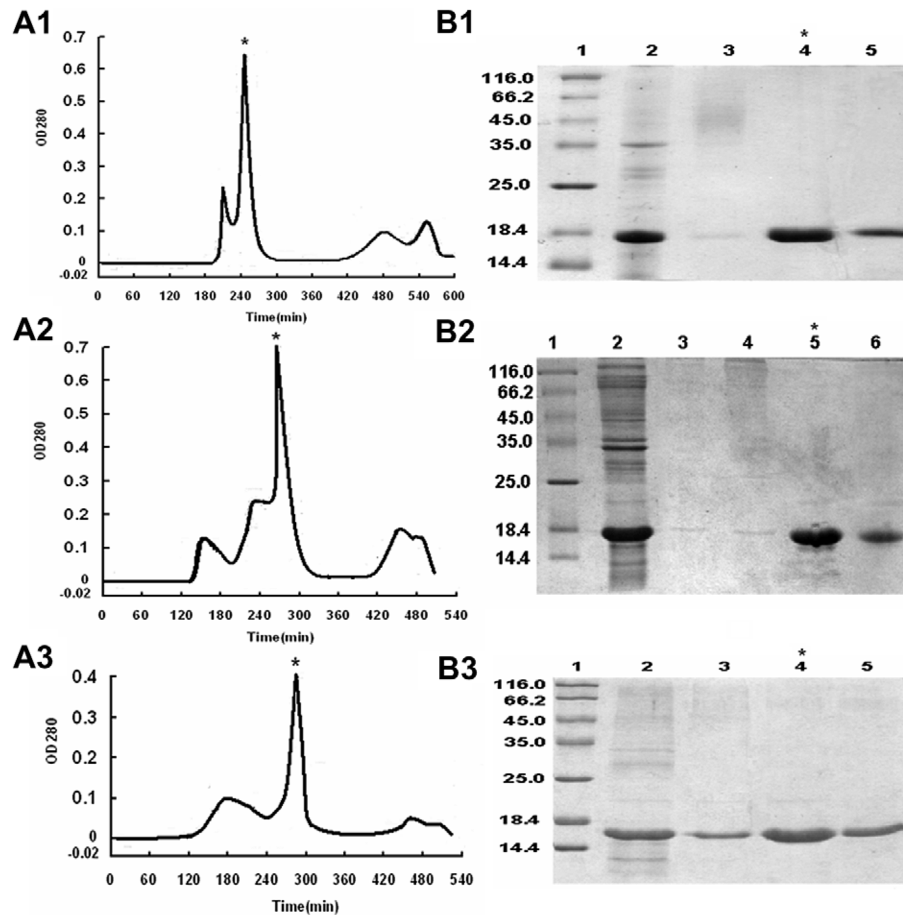


Fig. 3. Purification of mTNF mutants by size-exclusion chromatography. (A1, A2, and A3) Elution profile of mTNF mutants (Sephacryl-100 or Sephacryl-300). A chromatography column (2.4 × 70 cm) was packed with 350 ml of depyrogenated Sephacryl S-100 resin. Another chromatography column (2.4 × 100 cm) was packed with 500 ml of depyrogenated Sephacryl-300 resin. The column was equilibrated with 50 mM Tris–Cl, pH 8.5, 8 M urea, and 150 mM NaCl. The supernatant of solubilized mTNF mutant was applied to the column. The chromatography was performed at a flow rate of 0.3 ml/min. The mTNF mutant fractions were eluted with equilibration buffer. The peak * is the target protein. (B1, B2, and B3) SDS–PAGE analysis of mTNF-α mutants after purification by Sephacryl S-100. The lane * is the target protein.

mice. A lethal dose of LPS (600 μg) was injected intraperitoneally in TNF-PADRE vaccinated mice ($n = 10$), nonvaccinated controls ($n = 10$) and anti-mTNFα serum treated mice ($n = 10$). The survival time of the mice was determined (Fig. 6B). Statistical analyses of the differences among vaccinated, nonvaccinated and anti-mTNFα serum treated mice were performed using log-rank test. Survival rates analysis showed that both anti-mTNF-α serum and TNF-PADRE can prolong survival times in the endotoxic shock mice (TNF-PADRE and anti-mTNFα serum group $P < 0.05$ vs. saline group). TNF-PADRE autovaccine and anti-TNF serum have similar result in prolong survival times in the endotoxic shock mice (TNF-PADRE group $P > 0.05$ vs. anti-mTNFα serum group). The experiments were blinded and repeated four times.

Daily injections of mTNF-α ($1 \times 10^5$ IU) into BALB/c mice led to animals lost up to 18% of their body weights in control group. However, the body weights of mice vaccinated by TNF-PADRE only lost 5% of their initial body weight (Fig. 6D). The weight loss was reduced at least three times in vaccinated mice compared with nonvaccinated control. After daily injections of mTNF-α ($2 \times 10^5$ IU) into BALB/c mice twice, the mortality of nonvaccinated group reached to 100%, whereas the mortality of TNF-PADRE vaccinated group only was 50% (Fig. 6C). The experiments were blinded and repeated four times with similar results. Statistical analyse of the difference between vaccinated and control mice was performed using log-rank test. The immunization with mTNF-PADRE without adjuvant could prolong survival terms in BALB/C mice induced cachexia by mTNF-α (TNF-PADRE group $P < 0.05$ vs. saline group).

## 4. Discussion

At present, one obstacle in the development of a protein autovaccine is immunological T cell tolerance against a self-antigen. T cell tolerance to the native self-protein could be broken down after a foreign immunodominant T helper cell epitope is inserted into it. However, it is still important to choose more effective T helper cell epitope and proper gene location coupled with T helper cell epitope for the

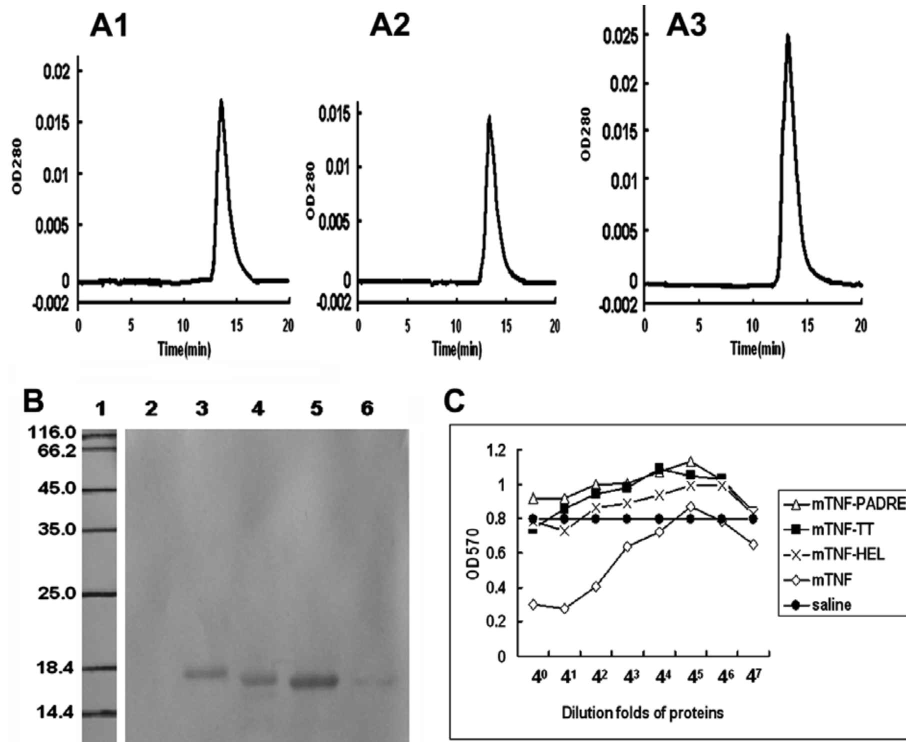


Fig. 4. Characterization of mTNF-TT, mTNF-HEL and mTNF-PADRE, Western blot and the bioactivity. HPLC analysis of purified mTNF-TT (A1), mTNF-HEL (A2), and mTNF-PADRE (A3) after refolding. SEC-HPLC analyses were performed on a Beckman's HPLC system. The sample in PBS was injected onto a $7.5 \times 300$ mm G2000SW column (TOSOH Corporation) at a flow rate of 0.5 ml/min. Peaks were detected by monitoring at a wavelength of 280 nm. (B) Western blot analysis of mTNF-TT, mTNF-HEL, and mTNF-PADRE. Lane 1, molecular weight standards (kDa); lane 2, whole cell lysate of pBV220-mTNF-TT/DH5α before induction; lane 3, Purified mTNF-TT; lane 4, mTNF-HEL; lane 5, mTNF-PADRE; lane 6, positive control (purchased mTNF-α). (C) The bioactivity of renatured TNF-TT, TNF-HEL, and TNF-PADRE. L929 cells were incubated with samples which were serially diluted by 4-fold for 16 h. The initial concentration of mTNF-α was 1 pg/ml; the initial concentration of renatured TNF-TT, TNF-HEL, and TNF-PADRE was 100 μg/ml. Saline was used as a negative control.

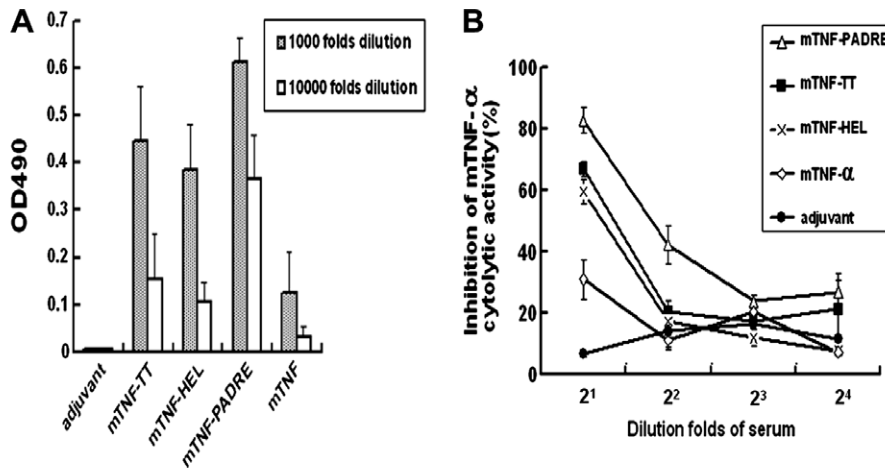


Fig. 5. The comparison of the antibody response induced by mTNF-TT, mTNF-HEL, and mTNF-PADRE. (A) Identification of serum anti-mTNF IgG reactivity by ELISA. The recombinant mTNF was used as the target antigen. Sera from the mice vaccinated by TNF-α mutants demonstrated high-titer (more than 1:10,000) specific antibodies against mTNF. The mTNF-PADRE induced the strongest anti-mTNF antibody response among the three proteins (at 1000- and 10,000-fold dilution, TNF-PADRE group $P < 0.05$ vs. TNF-TT group and $P < 0.01$ vs. TNF-HEL group.). (B) Identification of neutralization of mTNF-mediated cytotoxicity in L929 cells by the antiserum induced by TNF-α mutants. L929 cells were treated with series diluted antiserum, respectively. In the presence of mTNF (20 U/ml) for 16 h and cell survival was measured by crystal violet staining assay. The results showed that the antiserum induced by TNF-TT, mTNF-HEL, or mTNF-PADRE could neutralize 20 U/ml of mTNF in dose-dependent manner. At 2-fold dilution, the antiserum induced by TNF-TT, mTNF-HEL, and mTNF-PADRE could protect 66%, 59%, and 82% L929 cells against cytotoxicity induced by mTNF, respectively. The mTNF-PADRE induced antiserum showed the highest neutralizing ability (at 2- and 4-fold dilution, mTNF-PADRE group $P < 0.05$ vs. mTNF-TT and $P < 0.01$ vs. mTNF-HEL group). A representative result of three perform is shown.

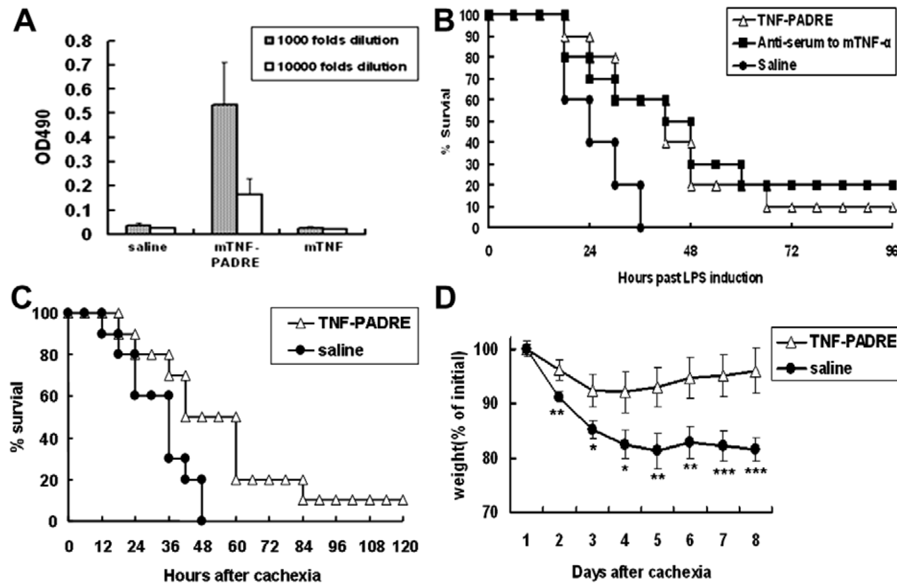


Fig. 6. The immunization with mTNF-PADRE without adjuvant ameliorated the symptoms of experimental endotoxic shock and cachexia in BALB/C mice. (A) ELISA analysis of antiserum from mice immunized with mTNF-PADRE without adjuvant. BALB/c mice ($n = 10$) were immunized with 30 μg of mTNF-PADRE without adjuvant four times. Two weeks after the last immunization, Specific serum antibodies against mTNF-α were detected by ELISA. The titer of lgG was over $10^4$. The serum from mice immunized with saline and mTNF without adjuvant was used as controls. (B) The immunization with mTNF-PADRE without adjuvant prolonged survival terms in endotoxic shock BALB/C mice induced by LPS. Two weeks after the last immunization, A lethal dose of LPS (600 μg, *E. coli* strain 055:B5) was injected intraperitoneally in TNF-PADRE vaccinated mice ($n = 10$), nonvaccinated controls ($n = 10$) and anti-mTNFα serum treated mice ($n = 10$). The survival time of the mice was determined (TNF-PADRE and anti-mTNFα serum group $P < 0.05$ vs. saline group, TNF-PADRE group $P > 0.05$ vs. anti-mTNFα serum group). (C) The immunization with mTNF-PADRE without adjuvant prolonged survival terms in mTNF induced cachexia in BALB/C mice. Two weeks after the last immunization, Appropriate dose ($2 \times 10^5$ IU) of biologically active mTNF-α was injected intraperitoneally daily in mTNF-PADRE immunized BALB/c mice ($n = 10$) and saline immunized BALB/c mice ($n = 10$). The survival rates of surviving mice were determined. Survival rates analysis showed TNF-PADRE can prolong survival terms in mTNF induced cachexia mice (TNF-PADRE group $P < 0.05$ vs. saline group.). (D) The immunization with mTNF-PADRE without adjuvant reduced the weight losses in mTNF induced cachexia in BALB/C mice. Two weeks after the last immunization, Appropriate dose ($1 \times 10^5$ IU) of biologically active mTNF-α was injected intraperitoneally daily in mTNF-PADRE immunized BALB/c mice ($n = 10$) and saline immunized BALB/c mice ($n = 10$). The body weights of mice were determined. Statistical analysis showed TNF-PADRE can reduced the weight losses in mTNF induced cachexia in BALB/C mice ($^{*}P < 0.005$, $^{**}P < 0.002$, $^{***}P < 0.001$). A representative result of four perform is shown.

construction of a protein autovaccine and the induction of high-titer neutralizing autobody.

In this study, how to choose the location of mTNF-α sequence, which is replaced by the T cell helper epitope, is crucial. We cannot choose N-terminal of mTNF-α because it is important for the receptor interaction [26,27]. We analyzed the potential antigenic sites on mTNF-α sequence by using the method of Kolaskar and Tongaonkar [28] and found that there were four zones of antigenic site in mTNF-α (amino acid residues: 10–21, 32–38, 111–125, 146–153). To break down the immunological tolerance to mTNF-α and not destroy the zones of antigenic site of mTNF-α, the sequence of amino acids (amino acid residues: 126–140) of mTNF-α was chosen and replaced by three T helper cell epitopes, respectively.

Although the three fusion proteins were successfully expressed in *E. coli*, they formed inclusion bodies. We develop a simple strategy to purify them using Sephacryl column. The final purity of the three proteins was above 95%. It is important to find an optimal renaturation condition of inclusion bodies for keeping immunogenicity of vaccine molecule. We have tested different pH (6.5, 7.5, and 8.5) for the refolding buffer, respectively. The results proved that pH 8.5 was optimal. Under this pH condition, more soluble refolded proteins were obtained.

The renature of three purified proteins was important for inducing antibodies. After the mice immunized with the proteins, all antisera from the mice could cross-reacted with both denatured and naïve mTNF-α. It suggested that the three proteins kept the proper native three-dimensional structure after they were refolded. Otherwise the neutralization autoantibodies could not be elicited in the mice. In addition, we also proved that the purified three proteins totally lost the cytolytic activity of native mTNF-α. Those data proved that preparation of our mTNF-α autovaccines is successful.

Although mTNF-TT, mTNF-HEL, and mTNF-PADRE elicited high-titer autoantibodies which bind with mTNF by ELISA. When antiserum was diluted eight times, all the serum almost lost their activity of neutralizing 20U of mTNF. However, in Nielsen's study [29], mice were immunized with purified human TNF variants and serum concentrations as low as 0.01–0.03% (corresponding to a serum dilution of 10,000–30,000) were sufficient to achieve 50% inhibition of human TNF-mediated cytotoxicity in KD4 cells. There are two possible reasons for low concen-

tration of the antiserum. One is that the induction of neutralization antibodies of foreign proteins was easier than that of self proteins. The other is that mouse posses an inner regulatory mechanism which prevent normal immune system from producing harmful TNF-α autoantibody.

To find which one was more effective among three T helper cell epitopes, the antiserum were analyzed. The result indicated that the titer from the mice immunize with mTNF-PADRE was the highest compared with others. PADRE is very effective epitope peptide. It is approximately 1000 times more powerful than natural T cell epitopes [19]. Our data suggests that PADRE is beneficial to help mTNF autovaccine eliciting high-titer antibody in vivo and may be a better epitope in the similar research.

In general, adjuvant is necessary in autovaccine autoimmunization development because it is difficult to elicit high-titer autoantibody. However, there are few adjuvants that were approved for human. In this study, we immunized the mice with mTNF-PADRE without adjuvant. We found that in the absence of adjuvant, mTNF-PADRE could also elicit high-titer anti-mTNF-α autoantibodies (>1:10,000), but mTNF-TT, mTNF-HEL only elicit low (<1:1000) (data not show). Then we used mTNF-PADRE without adjuvant to treat mTNF-α induced cachexia model. As a result, the mortality of the vaccinated mice was decreased by 50% after two days cachexia induction and the body weights of the mice were increased 3-fold in comparison with control mice.

LPS can induce experimental endotoxic shock. Most effects of LPS act via endogenous mediators, such as cytokines [30]. Among these cytokines, tumor necrosis factor-α (TNF-α) seems to be particularly important for endotoxic effects [31]. Antiserum to mTNF-α was useful to protect mice from LPS induced endotoxic shock [25]. However, at present, there are still no reports about using TNF-α autovaccine to treat LPS induced endotoxic shock. In this study, we used mTNF-PADRE to treat LPS induced endotoxic shock in the absence of adjuvant. We found that the survival time of the mice were prolonged compared with control. It demonstrated that the antiserum induced by mTNF-PADRE could neutralize overexpressed native mTNF-α induced by LPS in vivo. The antiserum to mTNF-α and mTNF-PADRE have comparable effects.

In summary, mTNF-PADRE we engineered could effectively elicited anti-mTNF-α antibodies and neutralize mTNF-α in vitro and vivo. PADRE is more powerful epitope for the similar research. Adjuvant is not necessary for the induction of TNF-α neutralizing antibody in this study. Recombinant mTNF-PADRE may be a better candidate of mTNF-α autovaccine.

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## References

[1] J. Nakashima, M. Tachibana, M. Ueno, A. Miyajima, S. Baba, M. Murai, Association between tumor necrosis factor in serum and cachexia in patients with prostate cancer, Clin. Cancer Res. 4 (7) (1998) 1743–1748.

[2] Y.Y. Wang, G.H. Lo, K.H. Lai, J.S. Cheng, C.K. Lin, P.L. Hsu, Increased serum concentrations of tumor necrosis factor-alpha are associated with disease progression and malnutrition in hepatocellular carcinoma, J. Chin. Med. Assoc. 66 (10) (2003) 593–598.

[3] S. Ganesan, S.P. Travis, T. Ahmad, R. Jazrawi, Role of tumor necrosis factor in Crohn's disease, Curr. Opin. Invest. Drugs 3 (9) (2002) 1297–1300.

[4] M. Marini, G. Bamias, J. Rivera-Nieves, C.A. Moskaluk, S.B. Hoang, W.G. Ross, et al., TNF-alpha neutralization ameliorates the severity of murine Crohn's-like ileitis by abrogation of intestinal epithelial cell apoptosis, Proc. Natl. Acad. Sci. USA 100 (14) (2003) 8366–8371.

[5] L.W. Moreland, Drugs that block tumor necrosis factor: experience in patients with rheumatoid arthritis, Pharmacoeconomics 22 (Suppl. 2) (2004) 39–53.

[6] K. Hamilton, E.W. Clair, Tumor necrosis factor-alpha blockade: a new era for effective management of rheumatoid arthritis, Expert Opin. Pharmacother. 1 (5) (2000) 1041–1052.

[7] W. Wu, T. Yamaura, K. Murakami, M. Ogasawara, K. Hayashi, J. Murata, et al., Involvement of TNF-alpha in enhancement of invasion and metastasis of colon 26-L5 carcinoma cells in mice by social isolation stress, Oncol. Res. 11 (10) (1999) 461–469.

[8] K.Y. Shin, H.S. Moon, H.Y. Park, T.Y. Lee, Y.N. Woo, H.J. Kim, et al., Effects of tumor necrosis factor-alpha and interferon-gamma on expressions of matrix metalloproteinase-2 and -9 in human bladder cancer cells, Cancer Lett. 159 (2) (2000) 127–134.

[9] R.O. Williams, M. Feldmann, R.N. Maini, Anti-tumor necrosis factor ameliorates joint disease in murine collagen-induced arthritis, Proc. Natl. Acad. Sci. USA 89 (20) (1992) 9784–9788.

[10] M.F. Neurath, I. Fuss, M. Pasparakis, L. Alexopoulou, S. Haralambous, K.H. Meyer zum Buschenfelde, et al., Predominant pathogenic role of tumor necrosis factor in experimental colitis in mice, Eur. J. Immunol. 27 (7) (1997) 7743–7750.

[11] H. Lochs, G. Adler, C. Beglinger, R. Duchmann, J. Emmrich, K. Ewe, et al., Anti-TNF antibody in Crohn's disease—status of information, comments and recommendations of an international working group, Z. Gastroenterol. 37 (6) (1999) 509–512.

[12] K.P. Machold, J.S. Smolen, Adalimumab—a new TNF-alpha antibody for treatment of inflammatory joint disease, Expert Opin. Biol. Ther. 3 (2) (2003) 351–360.

[13] R.L. Yung, Etanercept immunex, Curr. Opin. Invest. Drugs 2 (2) (2000) 216–221.

[14] R.O. Dillman, D.L. Shawler, T.J. McCallister, S.E. Halpern, Human anti-mouse antibody response in cancer patients following single low-dose injections of radiolabeled murine monoclonal antibodies, Cancer Biother. 9 (1) (1994) 17–28.

[15] P.M. LoRusso, P.L. Lomen, B.G. Redman, E. Poplin, J.J. Bander, M. Valdivieso, Phase I study of monoclonal antibody-ricin a chain immunoconjugate Xomazyme-791 in patients with metastatic colon cancer, Am. J. Clin. Oncol. 18 (4) (1995) 307–312.

[16] G.W. Philpott, S.W. Schwarz, C.J. Anderson, F. Dehdashti, J.M. Connett, K.R. Zinn, et al., RadioimmunoPET: detection of colorectal carcinoma with positron-emitting copper-64-labeled monoclonal antibody, J. Nucl. Med. 36 (10) (1995) 1818–1824.

[17] I. Dalum, D.M. Butler, M.R. Jensen, P. Hindersson, L. Steinaa, A.M. Waterston, et al., Therapeutic antibodies elicited by immunization against TNF-alpha, Nat. Biotechnol. 17 (7) (1999) 666–669.

[18] A.M. Waterston, F. Salway, E. Andreakos, D.M. Butler, M. Feldmann, R.C. Coombes, TNF autovaccination induces self anti-TNF antibodies and inhibits metastasis in a murine melanoma model, Br. J. Cancer 90 (6) (2004) 1279–1284.

[19] J. Alexander, J. Sidney, S. Southwood, J. Ruppert, C. Oseroff, A. Maewal, et al., Development of high potency universal DR-restricted helper epitopes by modification of high affinity DR-blocking peptides, Immunity 1 (9) (1994) 751–761.

[20] A.M. Waterston, L. Gumbrell, T. Bratt, Ÿ.S. Waller, J. Gustav-Aspland, Ÿ.C. L' Hermenier, et al., Phase I study of TNFa autovaccine in patients with metastatic cancer, Cancer Immunol. Immunother. 54 (9) (2005) 848–857.

[21] Z. Zhang, L. Yao, Y. Hou, Construction and application of a high level expression vector containing PR PL promoter, Chin. J. Virol. 6 (2) (1990) 111–116.

[22] X. Xue, Z. Wang, Z. Yan, J. Shi, W. Han, Y. Zhang, Production and purification of recombinant human BLyS mutant from inclusion bodies, Protein Expr. Purif. 42 (1) (2005) 194–199.

[23] D.A. Flick, G.E. Gifford, Comparison of in vitro cell cytotoxic assays for tumor necrosis factor, J. Immunol. Methods 68 (1–2) (1984) 167–175.

[24] A.M. Badger, D. Olivera, J.E. Talmadge, N. Hanna, Protective effect of SK&F 86002, a novel dual inhibitor of arachidonic acid metabolism, in murine models of endotoxin shock: inhibition of tumor necrosis factor as a possible mechanism of action, Circ. Shock 27 (1) (1989) 51–61.

[25] B. Beutler, I.W. Milsark, A.C. Cerami, Passive Immunization against cachectin/tumor necrosis factor protects mice from lethal effect of endotoxin, Science 229 (4716) (1985) 869–871.

[26] M.J. Eck, S.R. Sprang, The structure of tumor necrosis factor-alpha at 2.6 A resolution. Implications for receptor binding, J. Biol. Chem. 264 (29) (1989) 17595–17605.

[27] J.W. Bloom, J.D. Bettencourt, G. Mitra, Epitope mapping and functional analysis of three murine IgG1 monoclonal antibodies to human tumor necrosis factor-alpha, J. Immunol. 151 (5) (1993) 2707–2716.

[28] A.S. Kolaskar, P.C. Tongaonkar, A semi-empirical method for prediction of antigenic determinants on protein antigens, FEBS Lett. 276 (1-2) (1990) 172–174.

[29] F.S. Nielsen, J. Sauer, J. Backlund, B. Voldborg, K. Gregorius, S. Mouritsen, T. Bratt, Insertion of foreign T cell epitopes in human tumor necrosis factor-α with minimal effect on protein structure and biological activity, J. Biol. Chem. 279 (32) (2004) 33593–33600 (Epub 2004, Jun. 1).

[30] R. Takemura, Z. Werb, Secretory products of macrophages and their physiological functions, Am. J. Physiol. 246 (1Pt. 1) (1984) C1–C9.

[31] M.J. Jansen, T. Hendriks, M.T. Vogels, J.W. van der Meer, R.J. Goris, Inflammatory cytokines in an experimental model for the multiple organ dysfunction syndrome, Crit. Care Med. 24 (7) (1996) 1196–1202.